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Sequence requirements for localization and packaging of Ty3 retroelement RNA

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Abstract

Retroviruses and retrotransposons package genomic RNA into virus-like particles (VLPs) in a poorly understood process. Expression of the budding yeast retrotransposon Ty3 results in the formation of cytoplasmic Ty3 VLP assembly foci comprised of Ty3 RNA and proteins, and cellular factors associated with RNA processing body (PB) components, which modulate translation and effect nonsense-mediated decay (NMD). A series of Ty3 RNA variants were tested to understand the effects of read-through translation via programmed frameshifting on RNA localization and packaging into VLPs, and to identify the roles of coding and non-coding sequences in those processes. These experiments showed that a low level of read-through translation of the downstream open reading frame (as opposed to no translation or translation without frameshifting) is important for localization of full-length Ty3 RNA to foci. Ty3 RNA variants associated with PB components via independent determinants in the native Ty3 untranslated regions (UTRs) and in *GAG3-POL3* sequences flanked by UTRs adapted from non-Ty3 transcripts. However, despite localization, RNAs containing *GAG3-POL3* but lacking Ty3 UTRs were not packaged efficiently. Surprisingly, sequences within Ty3 UTRs, which bind the initiator $tRNA^{Met}$ proposed to provide the dimerization interface, were not required for packaging of full-length Ty3 RNA into VLPs. In summary, our results demonstrate that Gag3 is sufficient and required for localization and packaging of RNAs containing Ty3 UTRs and support a role for *POL3* sequences, translation of which is attenuated by programmed frameshifting, in both localization and packaging of the Ty3 full-length gRNA.

Keywords

yeast; retrotransposon; retrovirus; assembly; virus-like particles; packaging; RNA processing bodies; retrosome

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1. Introduction

Trafficking and packaging of gRNA into VLPs is a central process in the assembly of retroviruses and LTR retrotransposons (Beckham & Parker, 2008; Cochrane et al., 2006; Johnson & Telesnitsky, 2010; Klein et al., 2007; Sandmeyer & Clemens, 2010; White & Lloyd, 2012). Among retroviruses, significant differences exist with respect to where VLPs form. Assembly is cytoplasmic for beta-retroviruses and primate foamy virus, and contrastingly occurs at the plasma membrane for alpha- and gamma-retroviruses and lentiviruses. Even among retroviruses that undergo final assembly at the membrane, identification of gRNA is not constrained to a common compartment. *Saccharomyces cerevisiae* copia-like Ty1 and gypsy-like Ty3 retroelements are among the most extensively studied for LTR retrotransposon VLP assembly (Sandmeyer, 2002; Voytas & Boeke, 2002). These Ty elements assemble intracellularly in concentrations of retrotransposon proteins, gRNA and host RNA binding proteins, which we refer to as retrosomes (Beliakova-Bethell et al., 2006; Checkley et al., 2010; Dutko et al., 2010; Malagon & Jensen, 2008; Sandmeyer & Clemens, 2010). The current study was undertaken in order to identify the *cis*-acting sequences in the Ty3 LTR retrotransposon gRNA that stipulate delivery to the retrosome assembly site and packaging into VLPs.

1.1 Yeast Ty gRNAs

S. cerevisiae LTR retrotransposons Ty1-5 are ancient retrovirus-like elements which have proliferated throughout metazoans (Sandmeyer, 2002; Voytas & Boeke, 2002). Ty elements share their overall genome and gRNA structure with retroviruses in that the U3RU5 segments composing each LTR and intervening sequences are transcribed into a gRNA with RU5-*GAG-POL*-U3R structure. Copia-like Ty1, 2, 4, and 5 and gypsy-like Ty3 are only distantly related to one another. Both retrotransposons, similar to retroviruses, utilize translational frameshifting to produce a Gag-Pol polyprotein precursor at a level less than one-twentieth that of free Gag (Farabaugh, 1995; Farabaugh et al., 1993; Kirchner et al., 1992). Ty1 Gag-p49 is processed into CA-p45 and Gag-p4, and Gag-Pol is processed into CA-p45, PR, IN and RT domains (Voytas & Boeke, 2002). The Gag-p4 sequence also serves as the N-terminal portion of PR. While Ty1 does not have a canonical, retroviral-like NC domain containing a zinc finger motif, other domains of Gag and Gag-Pol contribute NC-like functionalities: a basic region of CA-p45 has RNA binding capability (Cristofari et al., 2000), and the Gag-p4 portion of PR is necessary for reverse transcription (Lawler et al., 2002). In contrast, the genome organization of Ty3 is more closely related to animal retroviruses. Ty3 Gag3-p34 is processed into CA-p24, spacer-p3 and NC-p7. CA contains a MHR motif and NC contains one zinc-binding motif, CCHC, Gag3-Pol3 is processed into CA-p24, spacer-p3, NC-p9, PR, RT and IN domains (Hansen et al., 1992; Kirchner & Sandmeyer, 1993; Kuznetsov et al., 2005). Both Ty1 and Ty3 use initiator $tRNA^{Met}$ as the reverse transcription primer, and mutations introduced to the TΨC arm and acceptor stem of the primer tRNA disrupt transposition (Keeney et al., 1995). Ty1 has a bipartite PBS in the 5’ end of *GAG* (Friant et al., 1998); in contrast, Ty3 has a bipartite PBS split between the 5’ and 3’ UTRs (Gabus et al., 1998). Both Ty1 (Feng et al., 2000) and Ty3 (Nymark-McMahon et al., 2002) VLP gRNAs are dimeric, and Ty1 protease activity contributes to the stability of dimeric gRNA (Feng et al., 2000). Association of the initiator $tRNA^{Met}$ with gRNA is proposed, based on *in vitro* experiments, to provide the dimeric gRNA interface (Cristofari et al., 1999; Gabus et al., 1998).

Ty1 and Ty3 expression can be artificially regulated under the *GAL1-10* UAS to induce high levels of retrotransposition (Boeke et al., 1986; Hansen et al., 1988). *Cis*-acting sequences required for the intracellular packaging of retrotransposon gRNA into VLPs are challenging to define since VLPs can only be isolated from cell lysates and are heavily contaminated with cytoplasmic RNA. However, transposition assays using donor Ty

elements in which internal domains are substituted with genetic markers have delimited sequences sufficient in *cis* for retrotransposition within the terminal 200 nts of the gRNAs of both types of Ty elements (Kirchner et al., 1992; Xu & Boeke, 1990).

1.2 Ty assembly foci

Cells induced to express Ty3 protein visualized with anti-CA or GFP fused to Gag3-Pol3 and RNA tagged with a MS2-binding site and expressed in the presence of MS2-RFP show an early punctate staining pattern and within 1–2 h nuclear pore-associated foci containing Ty3 protein and RNA (Beliakova-Bethell et al., 2006; Hansen et al., 1992). A combination of observations indicates that these foci are centers of Ty3 assembly. First, electron microscopy shows that cells expressing Ty3 accumulate clusters of VLPs rather than isolated VLPs (Hansen et al., 1992); second, RNA and protein co-localize to foci shown by immuno-electron microscopy to correspond to VLP foci (Beliakova-Bethell et al., 2006); third, mutations in the CA or NC domain of *GAG3* that are predicted to affect multimerization or RNA binding disrupt formation of foci (Larsen et al., 2008; Larsen et al., 2007); and fourth, deletions of some host genes required for transposition disrupt foci formation at a point after protein synthesis (Beliakova-Bethell et al., 2006; Irwin et al., 2005) and *(manuscript in preparation)*.

Genome-wide screens for factors which support retrotransposition of Ty1 (Griffith et al., 2003; Nyswaner et al., 2008; Scholes et al., 2001) and Ty3 (Irwin et al., 2005) implicated PB components at steps downstream of transcription. Similar to animal cells, budding yeast PB foci contain components of the deadenylation-dependent 5’ to 3’ degradation apparatus, including decapping enhancers Edc3, Pat1, and DEAD-box helicase Dhh1 (hDDX6/p54); decapping factors Dcp1,2; and the 5’ to 3’ exonuclease Xrn1, among other proteins (Aizer et al., 2008; Anderson & Kedersha, 2009; Buchan et al., 2010; Kedersha & Anderson, 2007). PB composition overlaps that of SG. For example, Xrn1 and Dhh1 are found in both PB and SG foci (Swisher & Parker, 2010) and PB formation precedes and is physically associated with SG formation in yeast cells undergoing nutritional deprivation (Buchan et al., 2008; Hoyle et al., 2007).

Despite association of Ty proteins and RNAs with PB proteins, the relationship of Ty assembly foci to PBs is complex. Because Ty RNA and foci contain a combination of RNA granule components, we have adopted the term “retrosome” to refer to Ty assembly foci (Sandmeyer & Clemens, 2010). In the case of Ty1, PB proteins not only facilitate retrotransposition, and are required for cluster formation (Checkley et al., 2010; Malagon & Jensen, 2008). However, functionally Ty1 clusters appear to differ from PBs; for example, they do not enlarge upon glucose deprivation (Checkley et al., 2010). However, they can be adjacent to, or temporally overlapping with PB reporters Dhh1- and Dcp2-GFP (Dutko et al., 2010). Although Ty1 retrosomes are not congruent with PBs, deletion of Xrn1 slows turnover of a repressive Ty1 antisense RNA (Berretta et al., 2008; Matsuda & Garfinkel, 2009; Dijk et al., 2011) and decreases packaging of Ty1 RNA into VLPs (Dutko et al., 2010).

Examination of cells expressing Ty3 fused at the downstream end of *POL3* to RFP-coding sequence together with a PB marker (Lsm1-, Dcp2-, Xrn1-, Dhh1-, or Edc3-GFP) showed that, in contrast to Ty1 retrosomes, Ty3 retrosomes contain PB components (Beliakova-Bethell et al., 2006) and at least one SG component *(manuscript in preparation)*. Similar to Ty1, Ty3 requires PB proteins Dhh1 and Xrn1 for retrotransposition and deficiency in either factor affects Ty3 focus formation (Beliakova-Bethell et al., 2006; Irwin et al., 2005). We speculate that retrosomes promote Ty3 VLP assembly by sequestering gRNA from the translation apparatus, concentrating Ty3 proteins and host assembly factors, and mediating localization of large numbers of VLPs in the vicinity of the nuclear pore complex.

Intracellular assembly of LTR retrotransposon VLPs presents problems analogous to those encountered in retrovirus translation, packaging and Gag multimerization. However, Ty3 VLPs complete assembly and presumably some extent of reverse transcription, and become remodeled to enable nuclear entry within a single host cell. Results from previous studies have highlighted the role of Gag3 protein domains in packaging and assembly of Ty3 VLPs. Mutagenesis of Gag3 showed that the CA NTD is critical for assembly (Larsen et al., 2007). Gag3 with mutations in the MHR forms diffuse foci, and fails to assemble or package RNA efficiently (Larsen et al., 2007; Orlinsky et al., 1996). Neutralization of the acidic charge of the Gag3 spacer arrests assembly, suggesting that interactions between spacer and the basic NTD of NC help to drive condensation of the VLP (Clemens et al., 2011). Disruption of the NC zinc-binding motif results in loss of Ty3 RNA foci but does not completely abrogate Gag3-Pol3 foci and Gag3 aggregates form in the nucleus (Larsen et al., 2008). These observations suggest that cytoplasmic localization of Gag3 depends upon gRNA interaction. This raises the possibility that during the course of wt Ty3 induction, free Gag3 could exceed the available supply of gRNA, and redistribute to the nucleus.

Retrovirus gRNA—Experiments examining the effects of transcription or translation inhibition on retrovirus gRNA packaging versus virion production have demonstrated that retrovirus RNAs can be committed to packaging in the nucleus or in the cytoplasm, without or after translation. MoMLV gRNA forms a separate pool from translated RNA soon after synthesis (Levin et al., 1974; Levin & Rosenak, 1976) and RNAs which are co-transcribed are preferentially co-packaged (Flynn et al., 2004). RSV Gag shuttles to the nucleus and promotes gRNA export, consistent with gRNA designation in the nucleus (Parent, 2011). In contrast, for HIV-1 and HIV-2, inhibition of transcription or nuclear export decreases cytoplasmic RNA, which affects translation products and virion gRNA similarly, indicating that these gRNAs are drawn from a common pool (Dorman & Lever, 2000). Within the cytoplasmic pool, viruses may differ with respect to mode of genomic capture. HIV-2 encoding truncated Gag is not packaged efficiently *in trans*, indicating that assembly is potentially initiated co-translationally with a resulting *cis* bias (Griffin et al., 2001; Kaye & Lever, 1999). On the other hand, in the case of HIV-1, inhibition of translation resulted in rerouting of RNA to packaging, indicating that translation is not a pre-requisite (Butsch & Boris-Lawrie, 2000; Dorman & Lever, 2000) and reviewed in (Butsch & Boris-Lawrie, 2002).

Retrovirus gRNA packaging requires specific signals in the RNA, typically a cluster of stem-loops overlapping the 5’ UTR which are recognized by the NC domain of Gag [reviewed in (Cimarelli & Darlix, 2002; D'Souza & Summers, 2005; Darlix et al., 1995; Johnson & Telesnitsky, 2010)]. Retroviral genomes are duplex, and dimer formation prior to packaging could facilitate proper copy number by contributing to recognition of gRNA by Gag. For example, dimerization of MLV gRNA exposes a high-affinity NC binding site within psi (D'Souza & Summers, 2004). Mutations in this sequence disrupt packaging of gRNA *in vivo,* supporting a model in which Gag binds to two high affinity sites in psi that are exposed upon dimerization (Gherghe et al., 2010). Disruption of specific basic residues contained in MLV NC reduces the level of packaged dimers without increasing the level of packaged monomers, consistent with packaging of a dimeric species (Housset et al., 1993). A genetic analysis of HIV-1 gRNA encapsidation also supports a role for dimeric species in packaging. *In vivo* visualization of gRNA using fluorescently-tagged RNA-binding proteins indicates that the vast majority of VLPs contain RNA dimers (Chen et al., 2009; Chin et al., 2007). Strikingly, RNAs with complementary DIS are preferentially co-packaged (Moore et al., 2007; Ni et al., 2011). More recent NMR studies of HIV-1 RNA-Gag interactions suggest conformation-driven packaging could be similar to that proposed for MLV. The HIV-1 5’ leader sequence can exist in two conformations, one of which promotes dimerization and exposes high-affinity NC binding sites proposed to promote packaging

(Heng et al., 2012; Lu et al., 2011). Thus, dimerization emerges a s a potentially general mechanism for selection of gRNAs for packaging.

1.2 Translation and gRNA capture

Selection of retroelement gRNAs from cytoplasmic pools implies coordination of translation template and genome functions. Evidence suggests that translation factors can associate with RNAs during nuclear export and affect downstream packaging fates. For example, in mouse cells, HIV-1 packaging is inefficient, but can be rescued by expression of human CBC, which binds RNA during export (Cochrane et al., 2006). Although HIV-1 gRNAs can be translated, translation is not necessary for packaging into virions (Butsch & Boris-Lawrie, 2000; Nikolaitchik et al., 2006). The proximity of psi to the site of Gag translation initiation suggests that local Gag accumulation could trigger assembly. This is supported by the observation that newly-translated HIV-1 Gag can feedback and eventually inhibit ongoing translation. This sequence of events would be consistent with the proposed model of co-translational assembly (Anderson & Lever, 2006).

In vivo retrovirus Gag association with gRNA has been monitored in several kinds of experiments, but with some consistent themes that relate to observations in retrotransposon systems. Pulse-chase analysis of HIV-1 Gag utilizing a cycloheximide block coupled with biarsenical tetracysteine staining showed an early diffuse pattern followed by collection in the perinuclear region and endosomal trafficking to the cell surface (Perlman & Resh, 2006). FRET analysis used to detect direct interactions between Gag and gRNA, as well as immunofluorescence (Poole et al., 2005) showed an early punctate cytoplasmic pattern, followed by concentration of Gag and gRNA in a pericentriolar region, and subsequently cell surface localization. Expression of psi-minus gRNA resulted in a diffuse Gag pattern, indicating that Gag-RNA interaction is required for normal trafficking and localization. Consistent with this, mutations in the HIV-1 Gag zinc-binding motif that disrupt high-affinity binding to psi caused Gag to be diffuse and cytoplasmic rather than punctate and plasma membrane-associated (Grigorov et al., 2007). Similarly, expression of psi-minus MLV RNA resulted in diffuse patterns of Gag (Basyuk et al., 2005; Basyuk et al., 2003), indicating that functional association of Gag with gRNA in the cytoplasm enables correct localization to the assembly site.

Thus, Gag is translated and in association with gRNA collects at the microtubule organizing complex, likely with newly-exported RNA for delivery via endosomal trafficking to the plasma membrane (Cochrane et al., 2006; Klein et al., 2007). Data are consistent with a model in which gRNAs undergo early cytoplasmic translation and potentially initiate assembly co-translationally (Anderson & Lever, 2006; Balvay et al., 2007; Griffin et al., 2001). For lenti-, gamma and some alpha retroviruses, assembly is completed at the plasma membrane, where a myristate moiety conjugated to the Gag amino-terminus and the basic region of Gag combine to stabilize Gag in the membrane, thereby allowing completion of multimerization and assembly (Cimarelli & Darlix, 2002).

1.3 Formation of HIV-1 gRNA ribonucleoprotein complexes

As discussed above, data from multiple systems supports co-trafficking of Gag and gRNA to the plasma membrane. Although Gag multimerizes upon RNA binding, there is less agreement regarding the level of this multimerization and nature of host factors within these RNPs. Some reports suggest that only low-level Gag multimers (dimers and trimers) are associated with gRNA prior to membrane delivery (Kutluay & Bieniasz, 2010). High-throughput siRNA screens (Brass et al., 2008; Bushman et al., 2009), mass spectrometric protein analysis (Mouland et al., 2001; Ott, 2008), biochemical assays (Bolinger et al., 2007) and fluorescence visualization of tagged candidate proteins have implicated host proteins in

HIV-1 gRNA trafficking. In addition to CRM1 and CBC, HIV-1 RNA export requires DDX3 (Yedavalli et al., 2004) and hnRNP A2 (Levesque et al., 2006; Mouland et al., 2001). *In vitro* studies led to identification of the ATPase ABCE1 as a host co-factor required for HIV-1 preassembly through a series of 80S, 150S, 500S complexes (Dooher et al., 2007). The double-stranded RNA-binding protein Staufen (Abrahamyan et al., 2010) has also been identified as a component of the HIV-1 gRNA-protein granule. During HIV-1 infection, Staufen gRNA-protein granule formation coincides with a decrease in the abundance of stress granule and PB-related protein complexes (Abrahamyan et al., 2010).

Components of RNA PBs, in addition to being implicated in Ty VLP assembly, have recently been identified as both co-factors and restriction factors for HIV-1 assembly. Animal cell PBs, similar to *S. cerevisiae*, contain deadenylation, decapping, 5' exonuclease and NMD- related proteins, but in contrast to *S. cerevisiae*, also contain eukaryotic RISC components (Anderson & Kedersha, 2009; Buchan et al., 2010; Moser & Fritzler, 2010; Thomas et al., 2011). Two recent investigations showed localization of HIV-1 RNA with PB components and identified miRNAs that antagonized HIV-1 replication. Consistent with host restriction activity, there were positive effects on HIV-1 replication of knockdown of RISC and PB components DDX6 (yeast Dhh1), Dcp2 and Ago3 (Chable-Bessia et al., 2009; Nathans et al., 2009). However, the role of PB-associated proteins is likely complex. The PB DEAD box helicase Mov10 associates with the RISC complex and is reported to modulate the activity of the subunit Ago2 in human cells (Liu et al., 2012). Overexpression decreased production of infectious exogenous retroviruses including HIV-1 (Burdick et al., 2010; Furtak et al., 2010; Wang et al., 2010) and LINE and Alu retrotransposons (Arjan-Odedra et al., 2012). Knockdown of endogenous levels of Mov10 has modest effects on exogenous retroviruses and reports are mixed as to the nature of those effects. However, consistent with the overexpression studies, knockdown of Mov10 increased retrotransposition of endogenous elements (Arjan-Odedra et al., 2012). Two other PB proteins, DEAD box helicase DDX6 and Dcp2, are associated with HIV-1 ABCE1 assembly complexes. DDX6 depletion reduces HIV-1 particle production, but not gRNA incorporation into assembled particles (Reed et al., 2012). DDX6 is also associated with primate foamy virus (PFV) Gag viral RNA and localizes with them at the peri-microtubule organizing complex assembly site (Yu et al., 2011). However, this case appears different from HIV-1 since PFV is not associated with the canonical PB marker Dcp1, and depletion of DDX6 reduced PFV gRNA packaging rather than particle production.

In the current study we set out to understand the roles of *cis*-acting, untranslated (UTRs) and translated (*GAG3-POL3*) regions in Ty3 RNA localization with PB reporters. Because PB proteins implicated in Ty3 retrotransposition act as modulators of translation and RNA stability, we also explored the relationship between translation and assembly foci. We found that Ty3 gRNA localization is sensitive to the state of gRNA translation and that RNAs with Ty3 UTRs and *GAG3-POL3* flanked by heterologous UTRs (hUTRs) localize independently to foci with PB proteins but only the former is packaged into VLPs. Surprisingly, packaging was not sensitive to mutations deleting the region proposed to mediate dimerization.

2. Methods

2.1 Yeast and bacterial culture conditions

Yeast and bacterial culture methods were as previously described (Amberg, 2005; Ausubel, 2002) except where noted. Bacterial strains HB101 [F^- *hsd-20* ($r_B^- m_B^-$) *recA13 leuB6 ara14 proA2 lacY1 galK2 rpsL20* (Sm^r) xyl-5 myl-1 supE44λ] or DH5α [F^- Φ80*lacZ*Δ *M15*Δ (*lacZYA-argF*) *U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1* λ^-] were used for plasmid preparations. Ty3 or *lacZ* RNAs tagged with the

MS2 binding site and MS2-RFP (Beliakova-Bethell et al., 2006) were expressed in strains derived from BY4741 (*MAT***a** *his3*Δ*1 leu2*Δ*0 met15*Δ*0 ura3*Δ*0*), by fusion of the genomic copy of *DHH1* or *DCP2* in-frame to the sequence encoding *GFP(S65T)* (Huh et al., 2003). Strain BY4741 *trp1*Δ was used for experiments with donor and helper plasmids. For 6 h packaging assays, a Ty3 null strain (*ygr109w*Δ::loxP *yil080w*Δ::loxP) was used. This strain was derived from BY4741 and constructed using loxP knock-out cassette amplification (Fang et al., 2011) with primers F5'-TCTGCTGCGCTACCACTGCGCCATACGAGCTTGATTTTCTGAAAGCGACTCTAGA G GATC-3' and R5'-TATAGTCATATTCCCATAAATAGAGCATCCAAAATGGAA-3' for *YGR109W* and F5'-ACAGGGTTTGTAGCACTGGTATACGTTTTTCCATATCATGGAACACGACTCTAG AGGATC-3' and R5'-GCCTTAACCAACTGGGCCAAGAGACCAGATATTTCATATGTTCTGATTCGAGCT CGGTAC-3' for *YIL080W*. To select for cells with particular prototrophic markers, synthetic dextrose medium [0.67% yeast nitrogen base-2% dextrose], synthetic galactose medium [0.67% yeast nitrogen base-2% galactose], or synthetic raffinose medium [0.67% yeast nitrogen base-1% raffinose-2% (vol/vol) glycerol-2% (vol/vol) lactic acid] containing complete amino acids, inositol and adenine sulfate lacking selection nutrients was used (Amberg, 2005).

2.2 Recombinant DNA constructions

Plasmids used in this study are summarized in Table 1 and Ty3 variants are illustrated in Fig. 1. Ty3 expression vectors were based on either pDLC201 (Hansen et al., 1988) or pYES2.0 (Invitrogen Corp., Carlsbad, CA). The pDLC201 plasmid is a high-copy, *URA3*-marked plasmid in which the *GAL1-10* UAS, oriented as it is in the *GAL10* promoter, is fused to Ty3 at nt 123 of the LTR (i.e. between U3 and R) conferring galactose regulation of the native Ty3 transcript. Although micro-heterogeneity is observed for the native transcript, the majority of transcripts have 5' and 3' UTRs 193 and 227 nt in length, respectively (Hansen et al., 1988). The pSBS2183 plasmid (previously pTy3-MS2) is pDLC201 modified by insertion of two copies of the MS2-binding site downstream of *POL3* to allow visualization of the Ty3 RNA (Beliakova-Bethell et al., 2006). Variants based on pSBS2183 express transcripts with the native Ty3 UTRs except for the presence of the MS2 sequence. The pYES2.0-based Ty3 expression plasmids are also high-copy and *URA3*-marked. Galactose-inducible Ty3 transcription initiates under regulation of the *GAL1* promoter and UAS and terminates under control of the *CYC1* terminator. In the pYES2.0 context, Ty3 transcripts have 5' *GAL1* (123–129 nts in length) and 3' *CYC1* (323–338 nts in length) UTRs. The expression plasmids and sequences of promoter, coding and terminator regions for each variant are provided in Supplemental Materials.

2.3 RNA and Dhh1 and Dcp2 reporter localization

MS2-tagged Ty3 RNAs (Ty3-MS2) were expressed in the presence of MS2 capsid protein fused to RFP (MS2-RFP) as described previously (Beliakova-Bethell et al., 2006). Cells were initially visualized using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss Inc.). A subset of these cells was then quantitatively analyzed using confocal fluorescence microscopy. BY4741 cells transformed with Ty3-MS2 and MS2-RFP expression plasmids were grown at 24°C in synthetic raffinose lacking Leu and Ura to late logarithmic phase, diluted in the same medium and grown overnight to OD_{600}= 0.2–0.3. Galactose was added to a final concentration of 2% in order to induce expression of Ty3 and cells were grown for 6 h at 24° C. At 30 min prior to live-cell imaging, cells were transferred to fresh medium of the same type, but lacking Met to induce MS2-RFP expression. Confocal laser scanning microscopy was performed on an inverted LSM510 laser scanning microscope (Carl Zeiss, Göttingen, Germany) using an oil immersion Plan-Apochromat 100×/1.4 NA objective. For

the localization of RFP (Ty3 mRNA) light from a helium-neon laser at 543 nm was directed over a HFT KP 700/543 beam splitter and fluorescence was detected using an NFT 545 beam splitter in combination with a BP 565–615 nm band pass filter; for localization of GFP (PB proteins), light from a 488 nm argon laser was directed over a HFT488 beam splitter in combination with a BP 500–550 nm band pass filter. The pinhole was set to 1 Airy unit. Laser power and detector sensitivity were optimized to avoid pixel saturation. Images were collected with a frame size of 1024 × 1024 pixels, and processed for publication with Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, CA). The experiments were repeated a minimum of two times with several independent transformants. The results were all similar. For quantitation and colocalization studies, single-plane confocal images from samples consisting of at least 150 cells from a single representative experiment were analyzed with Volocity Software 2.1 (PerkinElmer, Waltham, MA).

2.4 Ty3 RNA expression and nuclease protection assays

Ty3 RNA expression was quantified using a RT-qPCR assay. Expression of Ty3 RNA variants was induced as for microscopy, and 10 OD_{600} of cells was harvested. A Qiagen RNeasy kit was used to isolate total RNA according to the manufacturer's protocol. RNA was quantified using a Nano-drop spectrophotometer (Nanodrop Technologies, Inc. Wilmington, DE), and RNA was used to generate cDNAs for RT-qPCR analysis. Briefly, 1.5 μg total RNA was used as a template for cDNA synthesis using an oligo(dT) primer (final concentration of 0.025 μg/μL) in a Superscript II Reverse Transcriptase reaction according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The cDNAs were diluted 1:10 and used as template for qRT-PCR amplification. Two individual transformants were analyzed, and each cDNA sample was tested in triplicate. The following primers were used to amplify the gene targets of interest: Ty3 *GAG3*, F5'-CGAACTTGATGCTGATGGAGAC-3', R5'- GATCTTCTTGTCCTTACGGTATGG-3'; *ACT1*, F5'- ATTCTGAGGTTGCTGCTTTGG-3', R5'-TGTCTTGGTCTACCGACGATAG-3'. The amplification of each cDNA sample was monitored using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. The RT-qPCR cycles were performed using a RT-qPCR Cycler (Bio-Rad Laboratories, Hercules, CA) and the ratio of Ty3 *GAG3* to *ACT1* signal was calculated for each construct. *GAG3* and *ACT1* signals were compared to a standard curve to ensure that detection was within the linear range of the machine. Nuclease protection assays were performed with Benzonase (Sigma-Aldrich Corp., St. Louis, MO) or TurboNuclease (Accelagen, San Diego, CA) essentially as described (Clemens et al., 2011; Larsen et al., 2007).

2.5 Protein analysis

Yeast cells expressing wt or mutant derivative constructs were induced as for microscopy. Harvested cells were lysed under denaturing conditions to generate whole cell extracts as described previously (Clemens et al., 2011). Fifteen micrograms of total protein was subjected to SDS polyacrylamide gel electrophoresis and fractionated proteins were transferred to polyvinylidene fluoride membrane (Millipore Corp., Billerica, MA). Membranes were blocked with 2.5% non-fat milk in 1× PBST and incubated with primary rabbit polyclonal antibody against Ty3 CA (1:10,000), and primary mouse monoclonal antibody against Pgk1p (1:5,000) (Molecular Probes – Invitrogen, Carlsbad, CA). Protein bands were visualized with an ECL+ western blotting reagent kit (GE Healthcare, UK Ltd). A Fuji LAS-4000 imaging system and Multigauge V3.0 analysis software were used to quantify the chemiluminescent signal in each lane within the linear range of detection. Ty3 CA protein signal was normalized to the Pgk1p loading control signal for all samples, and mutant ratios were normalized to the wt Ty3 signal.

3. Results and Discussion

3.1 Ty3 UTRs and *POL3* act independently to promote localization of cytoplasmic Ty3 RNA with PB protein foci

One of the most distinctive features of cells expressing Ty3 is the formation of one or two large foci containing Ty3 protein and RNA, and PB components (Beliakova-Bethell et al., 2006). The current study was undertaken to define the roles of Ty3 RNA non-coding and coding sequences in this process. To this end, a set of Ty3-derived RNAs (Fig. 1) tagged downstream of the Ty3 coding region with the bacteriophage MS2 capsid protein binding site were expressed under control of the *GAL1-10* UAS in cells expressing MS2-RFP (Beliakova-Bethell et al., 2006). For simplicity, Ty3 RNA regions beginning at the 5' end from RU5 through to the initiator ATG and the 3' U3R region (containing a short portion of *POL3*) are referred to as UTRs. The contribution of Ty3 UTRs to gRNA function was assessed by substitution of Ty3 UTRs with hUTRs derived at the 5' and 3' ends from *GAL1 and CYC1,* respectively (Fig. 1). For the purpose of clarity, a construct name that does not contain a specific UTR designation represents a RNA variant that is flanked by hUTRs. The role of the *POL3* coding sequence was tested by deleting *POL3*, substituting a heterologous ORF for *POL3* and modulating the level of *POL3* translation. Transformants were evaluated for Ty3 RNA and protein expression, and confocal microscopy was performed to determine RNA localization by observing the number of foci greater than 0.1 μm^2 per MS2-RFP-positive cell and to determine overlap with Dhh1- and Dcp2-GFP reporter foci. Despite selection for Ty3 and MS2-RFP expression plasmids, only some cells are positive for both (Beliakova-Bethell et al., 2006) and *(data not shown)*.

Since differing levels of expression might affect localization with PB factors and focus formation, Ty3 protein and RNA in cells expressing the Ty3 constructs were quantified. Immunoblot analysis showed that levels of Ty3 Gag3 and processing products did not differ among transformants by more than two-fold (Fig. 2A). In cells expressing wt Ty3, programmed frameshifting results in production of Gag3 and Gag3-Pol3 in a ratio of about 20:1 (Kirchner et al., 1992). Cells expressing a Ty3 in which *GAG3* is fused to *POL3* (*GAG3*-FUS-*POL3*) do not make Gag3 through run-off translation of *GAG3*. However, Gag3-Pol3 is processed into a slightly longer Gag3 product (Kirchner et al., 1992; Orlinsky & Sandmeyer, 1994), which was present in amounts comparable to levels of wt Gag3 (Fig. 2A). These results indicated that for the most part similar amounts of Gag3 protein were generated from the constructs tested for RNA localization. Ty3 wt and variant RNA levels were quantified by RT-qPCR, using primers that annealed within *GAG3,* which was present in all constructs. Ty3 RNA levels were normalized to the level of *ACT1* RNA per cell (Fig. 2B). Most Ty3 RNA levels were within two-fold of the control. However, despite significant amounts of Gag3 produced, the normalized level of the RNA in which *POL3* was substituted with *lacZ* (*GAG3*-STOP-*lacZ*) was quite low compared to the normalized level of control Ty3 RNA (0.08 versus 1.20). Although the stop codon at the end of the *GAG3* ORF could have triggered NMD, a comparable decrease was not observed in constructs containing a stop codon downstream of the *GAG3* programmed frameshift site (UTR-*GAG3-POL3*-UTR and *GAG3*-STOP-*POL3*). Interestingly, despite similar levels of protein, RNA levels of UTR-*GAG3*-UTR and *GAG3* including and lacking Ty3 UTRs, were elevated by three- and two-fold, respectively, relative to control Ty3 RNA (Fig. 2A and B).

Cells expressing wt Ty3 RNA (UTR-*GAG3-POL3*-UTR) and Dhh1-GFP and Dcp2-GFP formed 0.71 and 0.87 Ty3 RNA foci per cell, respectively (Fig. 3). About half of the UTR-*GAG3-POL3*-UTR foci had an area of 0.5 μm^2 or greater. Variations in the size of MS2-RFP foci are most likely influenced by many factors including Ty3 RNA and protein levels. In addition, strains expressing different PB reporters, Dhh1-GFP and Dcp2-GFP showed differences in size of foci and in foci per cell. These differences could reflect effects of the

fluorescent reporter fusions on reporter function. Nevertheless, almost all MS2-RFP foci overlapped with Dhh1- and Dcp2-GFP reporter foci, indicating that the two PB reporters localized similarly (Fig. 3).

Ty3 UTRs are sufficient when supplied *in cis* to mediate retrotransposition of the marker gene *HIS3* (Kirchner et al., 1992). Therefore, we first tested the contribution of Ty3 UTRs to formation of RNA foci by comparing localization of wt UTR-*GAG3-POL3*-UTR RNA to RNA in which the native Ty3 5' and 3' UTRs were substituted with hUTRs (*GAG3-POL3*) (Fig. 3). *GAG3-POL3* RNA formed fewer foci with Dhh1-GFP (0.34 versus 0.71 foci per cell) and with Dcp2-GFP (0.67 versus 0.87 foci per cell) than did the native Ty3 RNA. However, *GAG3-POL3* RNA foci that did form co-localized with Dhh1- and Dcp2-GFP reporter foci similarly to wt Ty3 RNA (84 and 81%, versus 88% and 87%, respectively), and were similar in size distribution to those of wt Ty3 RNA (Fig. 3). These results clearly indicate that Ty3 UTRs promote inclusion of Ty3 RNA in foci with PB proteins. However, the result that *GAG3-POL3* lacking Ty3 UTRs localized to assembly foci was unanticipated.

Previous analysis of Gag3 mutants disrupted for T y3 RNA-binding demonstrated that Gag3 competent to bind RNA is required to form Ty3 RNA foci with PB proteins (Larsen et al., 2008). We therefore tested whether *POL3* was dispensable for focus formation by examining localization of RNAs lacking *POL3* with Ty3 UTRs (UTR-*GAG3*-UTR) and hUTRs (*GAG3*) compared to localization of RNAs containing *POL3*, wt Ty3 (UTR-*GAG3-POL3*-UTR) and *GAG3-POL3* with hUTRs (*GAG3-POL3*). Cells expressing UTR-*GAG3*-UTR showed a higher level of focus formation than cells expressing wt Ty3 (1.17 versus 0.71), but a similar or slightly lower level of coincidence with the PB reporter proteins (82% versus 88%, Dhh1-GFP and 73% versus 87%, Dcp2-GFP). Cells expressing *GAG3* with hUTRs showed diffuse fluorescence, and very few foci (0.04 and 0.18 foci per cell in the presence of the Dhh1- and Dcp2-GFP reporters, respectively). In addition, a lower percentage of these foci co-localized with PB reporters (71% and 41% for *GAG3* compared to 84% and 81% for *GAG3-POL3* with Dhh1- and Dcp2-GFP, respectively). Thus, in the full-length construct with hUTRs, *POL3* played an essential role in the localization of the RNA to foci and foci formed by RNAs containing *POL3* coincided to a greater extent with PB foci.

The *POL3* requirement for localization of *GAG3-POL3* RNA to foci with PB proteins prompted further exploration of the contribution of *POL3* to localization of Ty3 transcripts with Ty3 UTRs. As described above, programmed frameshifts are a prominent feature of retroelement gRNAs. As ribosomes translating Gag3 terminate approximately 20 times more frequently than reading through the programmed frameshift separating the two reading frames (Farabaugh et al., 1993; Kirchner et al., 1992), RNA downstream of the programmed frameshift site is expected to have lower ribosomal occupancy than *GAG3*. We first tested whether read-through translation into *POL3* and production of Gag3-Pol3 were important by introducing a stop codon downstream of the programmed frameshift between *GAG3* and *POL3* (UTR-*GAG3*-STOP-*POL3*-UTR). Compared to wt, cells expressing this construct showed fewer foci (0.49 versus 0.71 foci per cell). This result showed that Gag3-Pol3 protein is not essential for formation of Ty3 RNA foci with PB proteins. However, the lower number of foci per cell compared to those of wt UTR-*GAG3-POL3*-UTR suggested that RNA sequence of *POL3* alone did not constitute the basis of *GAG3-POL3* localization. The foci that formed with UTR-*GAG3*-STOP-*POL3*-UTR were somewhat larger than wt, and co-localized to a similar extent with the Dhh1- and Dcp2-GFP reporter foci. We hypothesized that either a low density of ribosomes in the *POL3* reading frame or specific sequences could promote localization by providing access to PB proteins. The possibility that low ribosome occupancy was important to focus formation was tested indirectly by fusing *GAG3* to *POL3* (UTR-*GAG*-FUS-*POL3*-UTR). This RNA formed fewer foci than

either UTR-*GAG3*-STOP-*POL3*-UTR or wt Ty3 (0.25 versus 0.49 or 0.71 foci per cell). However, foci that formed were similar in terms of size and co-localization with the Dhh1-GFP PB reporter (86% versus 88% or 88%). It was possible that the occurrence of programmed frameshifting might target Ty3 RNA for association with PB factors responsible for NMD. However, as discussed above, wt UTR-*GAG3*-*POL3*-UTR, UTR-*GAG3*-STOP-*POL3*-UTR and UTR-*GAG3*-FUS-*POL3*-UTR RNAs were present at comparable concentrations (Fig. 2B), suggesting that NMD was not a determining factor. Additional tests of *GAG3*-STOP-*lacZ* and *GAG3*-FUS-*POL3* showed dramatic decreases when low-level translation of the downstream reading frame was disrupted (0.11 and 0.12 foci per cell with the Dhh1 reporter). However, the former result was confounded by a low level of RNA (Fig. 2B). Thus, in the context of the Ty3 UTRs and full-length RNA, low ribosome occupancy of *POL3* could operate to allow focus formation in association with PB proteins. This is consistent with the ability of some of these proteins to associate with polysomes and down regulate translation [e.g. Lsm1-7, Pat1, and Dhh1 (Coller & Parker, 2004)].

The observations that inclusion of *GAG3* and *GAG3-POL3* RNAs in foci with PB proteins was significantly higher when coding sequences were flanked with Ty3 UTRs than when flanked with hUTRs indicates that Ty3 UTRs provide the dominant feature conferring inclusion of Ty3-related RNAs in foci with PB proteins. Positive-strand RNA viruses are characterized by relatively long and structured 5' UTRs, and, retroviruses rely on both cap-dependent and cap-independent mechanisms as well as specialized helicases to promote translation initiation (Balvay et al., 2007; Bolinger & Boris-Lawrie, 2009; Bushell & Sarnow, 2002). Retroelements may generally have features that antagonize translation, thereby promoting association of factors such as PB proteins. With a length of 181–193 nts, the Ty3 5' UTR is significantly longer than the average yeast 5' UTR (50 nts) (Nagalakshmi et al., 2008) and is predicted to be structured (*unpublished results*). Our quantification of RNA and protein produced from the *GAG3-POL3* and *GAG3* templates with Ty3 UTRs showed lower protein to RNA ratios than analogous transcripts with hUTRs (Fig. 2C). This suggests that Ty3 UTRs could function to antagonize translation and in that way promote PB protein association. However, other factors also appear to influence translation and packaging. In the absence of native UTRs, inclusion of *POL3* significantly enhanced the association of *GAG3*-containing RNA with PB proteins. Focus formation was diminished if the *POL3* ORF was either untranslated or fused to *GAG3* resulting in constitutive read-through translation. These data are consistent with roles for Gag3-Pol3 as well as Gag3 in focus formation. In addition, we hypothesize that frameshifting by attenuating the ribosomal occupancy of *POL3* could also promote PB protein association with Ty3 RNA. Our data do not exclude the possibility that specific sequences in the *POL3* region are important as binding sites for host factors involved in Ty3 RNA localization to foci.

3.2 Requirements for localization and packaging of *GAG3-POL3* RNAs differ

We next examined the relationship between Ty3 RNA localization to foci and packaging into VLPs. Packaging of retrotransposon gRNA into VLPs confers resistance to *in vitro* nuclease digestion (Lin & Levin, 1997) and protection increases with time of Ty3 induction (Clemens et al., 2011; Larsen et al., 2007) and (*unpublished data*). The packaging of Ty3 RNA with and without Ty3 UTRs was compared. After 24 h of Ty3 expression, 56% of wt Ty3 RNA (UTR-*GAG3-POL3*-UTR) was protected (Fig. 4A). *GAG3-POL3* RNA with hUTRs, which localized in foci with Dhh1- and Dcp2-GFP reporters, was significantly less protected than the native Ty3 RNA (17% versus 56%) (Fig. 4A). This result indicated that Ty3 gRNA localization into foci with Gag3 and PB proteins is not sufficient to insure wt levels of packaging; rather, one or both of the Ty3 UTRs are required.

As indicated above, low-level translation of *POL3* produced maximal focus formation. The negative effect of *GAG3-POL3* read-through translation on focus formation [compare in Fig. 3: UTR-*GAG3-POL3*-UTR and UTR-*GAG3*-FUS-*POL3*-UTR (0.71 versus 0.25 foci/cell) and *GAG3-POL3* and *GAG3*-FUS-*POL3* (0.34 versus 0.12 foci/cell)] suggested that ribosomal occupancy of the downstream ORF antagonized PB protein and Gag3 association with the gRNA, but that this was of greater consequence in the absence of the UTRs. In order to explicitly assess the effect of read-through translation on packaging, UTR-*GAG3*-STOP-*POL3*-UTR RNA was compared to UTR-*GAG3*-FUS-*POL3*-UTR RNA for protection from nuclease digestion. These experiments showed that UTR-*GAG3*-STOP-*POL3*-UTR was modestly more efficiently packaged than wt Ty3 RNA (66% versus 56%). In contrast, the UTR-*GAG3*-FUS-*POL3*-UTR RNA showed much less protection than wt (12% versus 56%) (Fig. 4A). While reduced retrosome formation is associated with this reduced protection, this construct has elevated Gag3-Pol3 to Gag3 ratios (Fig. 2A), and consequently may be reduced for polyprotein processing. In the case of HIV-1, reduced processing is associated with unstable dimers and reduced RNA stability (L'Hernault et al., 2012). Together, these results are consistent with the model that RNAs that associate more efficiently into foci with PB proteins are more readily packaged into VLPs. However, requirements for processed Gag3 also likely contributed to reduced packaging of UTR-*GAG3*-FUS-*POL3*-UTR RNA (Fig. 4A).

The experiments described above implicated one or both of the UTRs in packaging. The role of UTRs was tested by examining extracts of cells expressing UTR-*GAG3*-UTR, *GAG3* with 5’ and 3’ hUTRs, and *GAG3* with only the Ty3 5’ UTR or 3’ UTR (Fig. 3) and one hUTR. Because differences in packaging might be most apparent in the early phase after induction, protection was measured after 6 h rather than 24 h of induction. UTR-*GAG3*-UTR RNA, which localized efficiently to PB protein-containing foci was packaged to a much greater extent than *GAG3* with hUTRs (59% versus 17%) (Fig.4B). *GAG3* RNA with the 5’ UTR a lone was protected to a similar extent as RNA with both UTRs (61% versus 59%). *GAG3* RNA with only the Ty3 3’UTR was protected to a significantly lesser extent (33%), but still more than *GAG3* RNA with hUTRs (17%) (Fig. 4B).

In summary, these results argue that while Ty3 RNAs can be delivered to PB foci through independent pathways mediated by UTRs and *POL3*, Ty3 UTRs provide the key signal for productive packaging into VLPs. In the case of the *GAG3* RNA, both the Ty3 5’ and 3’ UTRs enhanced packaging, but the Ty3 5’ UTR had the greater effect. Independent of UTRs, translation of *POL3* correlated inversely with packaging. Lastly, although *GAG3* RNA with hUTRs represents a synthetic RNA that was less well packaged than UTR-*GAG3*-UTR, these results indicate that at least some packaging into Ty3 particles likely occurs in the absence of complete localization to macroscopic PB foci.

3.3 Importance of 5’-3’ bipartite primer binding sequences to Ty3 RNA packaging

Known retrovirus gRNA packaging sites contain DIS regions which contribute to packaging (Chen et al., 2009; Moore & Hu, 2009; Russell et al., 2004), consistent with the packaging of dimer species. In the case of Ty3, reverse transcription primer initiator $tRNA^{Met}$ has been shown to bind to a bipartite PBS in the 5’ and 3’ UTRs (Gabus et al., 1998). Furthermore, *in vitro* initiator $tRNA^{Met}$ primers bound to Ty3 gRNA have been proposed to anneal in anti-parallel orientation to each other at the tRNA 5’ ends and mediate mini-Ty3 dimerization (Gabus et al., 1998). Given a bipartite PBS, the ability of the 5’UTR alone to direct packaging of the *GAG3* RNA suggested that primer-mediated dimerization is dispensable for packaging. Full-length Ty3 RNA variants were constructed with the 5’ and 3’ PBS or 3’ PBS alone deleted from the UTRs to test the role of the bipartite PBS in packaging (Fig. 4C). Surprisingly, deletion of both the 5’ and 3’ segments of the bi-partite PBS or the 3’ PBS segment alone resulted in similar increases in protection to the full-length Ty3 (26%

and 25% versus 20%). Because we observed RNA protection in the absence of part or all of the bipartite PBS, our results suggest that if the tRNA primer bound to gRNA mediates dimerization of Ty3 gRNA *in vivo,* then, unlike what is observed for some retrovirus gRNAs, formation of the dimer interface is not a pre-requisite for Ty3 gRNA packaging. We also assayed these mutants for the ability to produce cDNA. As previously reported, disruption of the 5' PBS resulted in an inability to generate cDNA. As predicted by the bipartite priming model, and as suggested by in vitro experiments, the mutant that retained the 5' PBS but lacked the 3' PBS also did not produce cDNA (*unpublished results*).

3.4 Gag3 can mediate packaging of Ty3 UTR-containing RNAs *in trans*

In this study we found that the Ty3 RNA variant *GAG3* with Ty3 UTRs forms PB protein-associated foci and is protected against nuclease digestion, whereas *GAG3* with hUTRs is neither localized nor protected, thereby implicating UTRs in packaging. UTRs were previously shown to be sufficient to mediate transposition of a genetic marker if complemented *in trans* by expression of Ty3 proteins from a helper element (Kirchner et al., 1992) and (*unpublished data*), suggesting that Gag3 function can promote packaging of RNA *in trans*. Because the experiments in the current study examined constructs in which Gag3 was expressed *in cis,* an important outstanding question was whether Gag3 could localize RNA to PB protein foci when expressed *in trans*. *LacZ* flanked by heterologous or Ty3 UTRs was expressed in the presence or absence of Ty3 Gag3. *LacZ* formed visible foci only when flanked by Ty3 UTRs and in the presence of Gag3 (Fig. 5). These foci were less intense than when Gag3 was expressed in *cis*. Nevertheless, this demonstrated that in the presence of trans-acting Gag3, Ty3 UTRs mediate localization consistent with the previously observed ability of Ty3 expression to mediate donor transposition *in trans*.

4. Summary and Conclusions

Retroelement VLP assembly results from a balance of competing translation and packaging processes. We proposed previously that PB localization facilitates Ty3 gRNA packaging into particles by sequestering the gRNA from the translation apparatus (Beliakova-Bethell et al., 2006). Based on previous investigations and the current study, we propose that as Gag3 accumulates, it interacts with a packaging site contained in the Ty3 UTRs, thereby antagonizing translation and allowing association with PB proteins, which further antagonize translation. In addition, we hypothesize that *POL3,* because of specific sequences or reduced ribosomal occupancy or both, interacts with host factors including PB proteins and independently promotes development of retrosome foci. Host protein interaction with Ty3 proteins and RNA allow for RNA exit from translation and for concentration of Gag3, Gag3-Pol3, and RNA, thereby driving assembly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

aa	amino acids
ABCE1	ATP binding cassette sub-family E member 1
CA	capsid
CBC	cap binding complex
cDNA	copy DNA
Crm1	chromosome region maintenance protein 1
Dcp2	decapping protein 2
Dhh1/hDDX6	dead box helicase homolog/human DEAD (Asp-Glu-Ala-Asp) box polypeptide 6
DIS	dimerization initiation sequence
Edc3	enhancer of decapping protein 3
FRET	Förster (fluorescence) resonance energy transfer
GFP	green fluorescent protein
gRNA	genomic RNA
hUTRs	heterologous untranslated regions
HIV-1	human immunodeficiency virus
hnRNP A2	heterogeneous nuclear ribonucleoprotein A2
IN	integrase
Lsm1-7	Like-sm proteins 1-7
LTR	long terminal repeat
MHR	major homology region
miRNA	micro RNA
MLV	murine leukemia virus
Mov10	murine leukemia virus 10 protein
NC	nucleocapsid
NMD	nonsense-mediated decay
NMR	nuclear magnetic resonance
NTD	amino terminal domain
Pat1	protein associated with topoisomerase 1
PB	(RNA) processing body
PBS	primer binding site
PBST	phosphate buffered saline tween solution
PR	protease
Psi	packaging signal
RFP	red fluorescent protein
RISC	RNA induced silencing complex

RNP	Ribonucleoprotein
RSV	Rous sarcoma virus
RT	reverse transcriptase
SDS	sodium dodecyl sulfate
SG	stress granule
UAS	upstream
UTR	untranslated region
VLP	virus-like particle
WCE	whole-cell extract
Xrn1	exo-ribonuclease 1

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Highlights

- Ty3 UTRs or GAG3-*POL3* mediate RNA localization to Ty3 assembly foci.
- The Ty3 5’ UTR is sufficient for packaging of Ty3 *GAG3* or *GAG3-POL3* RNA into VLPs.
- The 5’-3’- bipartite PBS is not essential for packaging of Ty3 gRNA.

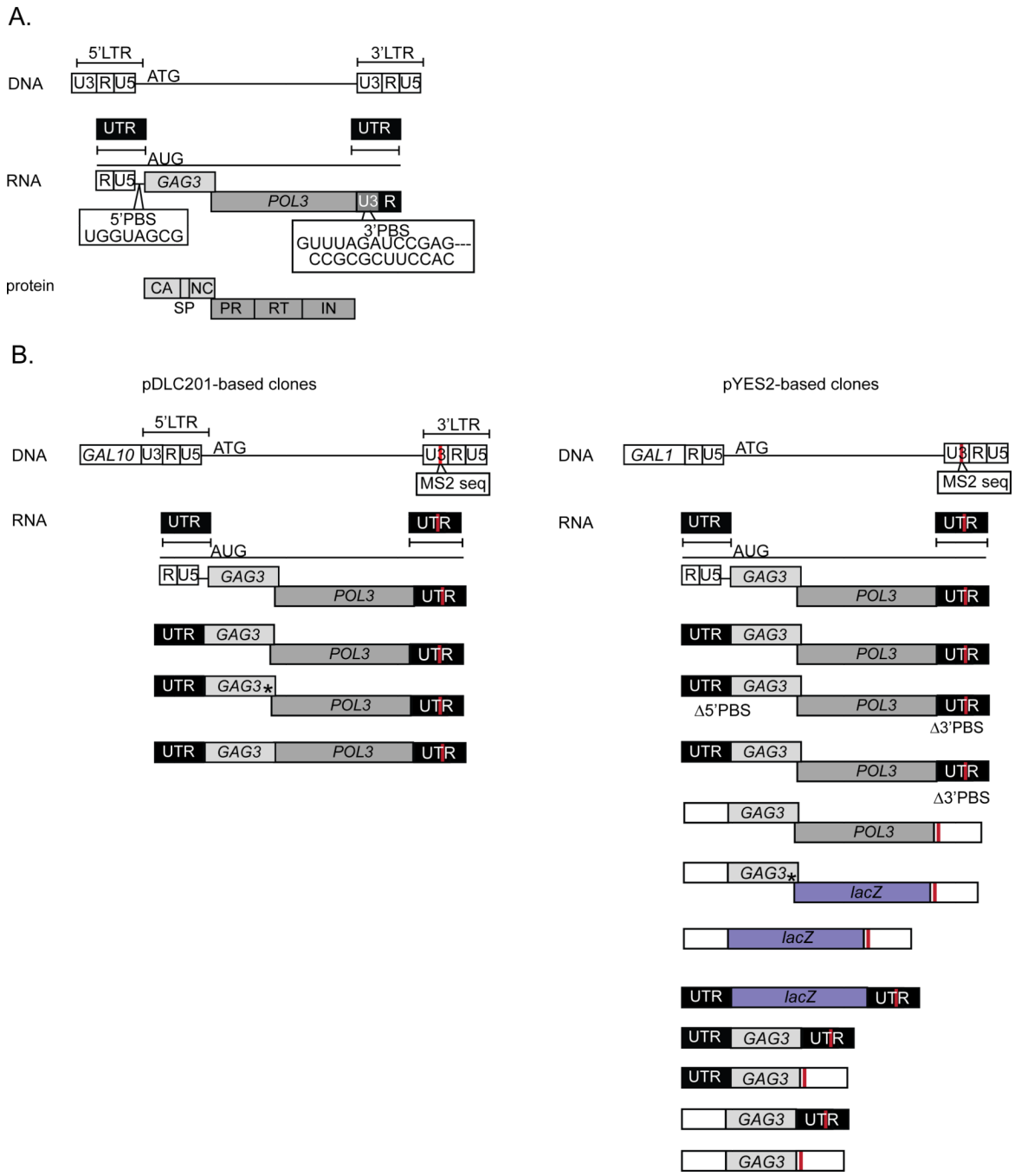

Figure 1. Ty3 genome and expression variants

A. Full-length Ty3 DNA (U3RU5-coding region-U3RU5) and RNA (RU5-coding region-U3R) sequences. First and second ORFs *GAG3* and *POL3* are translated into Gag3 and Gag3-Pol3 polyproteins and processed into the mature species indicated. The Ty3 gRNA 5'UTR is comprised of RU5 and 75 nt of the internal domain upstream of the initiator AUG. *POL3* extends into the downstream LTR by 46 nts. For simplicity in the text, Ty3 "UTRs" refers to the 5' UTR and the complete 3' U3R (of which 46 nts is translated). **B.** Ty3-related expression constructs. The *GAL1-10* UAS (*GAL10* orientation) partially replaced U3 in the upstream LTR to allow galactose-regulated expression of Ty3-related transcripts (left-hand

column). *Indicates stop codon. Right-hand column displays pYES2.0-based full length Ty3, *GAG3 and LacZ* reporter expression constructs. pYES2.0-based constructs containing a 5' UTR derived from Ty3 have the RU5 sequence placed downstream of the *GAL1* promoter. hUTRs were derived by transcription from pYES2.0 under control of the *GAL1-10* UAS, the *GAL1* promoter and 5' UTR, and the *CYC1* terminator. Empty white boxes denote hUTRs. Complete annotated plasmid sequences are presented in Supplemental Materials.

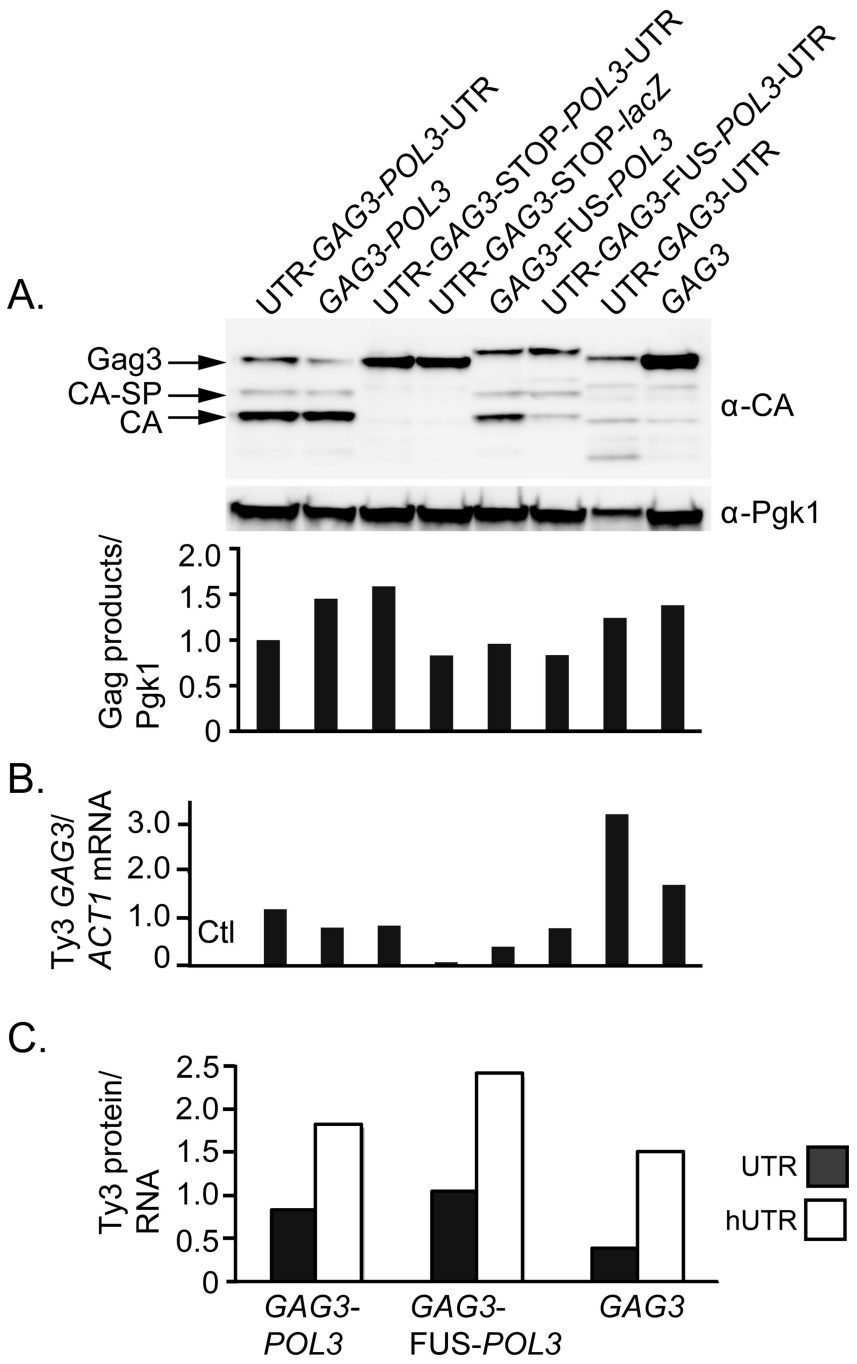

Figure 2. Expression of Ty3-related RNAs and proteins

A. Ty3 Gag3 expression. Total Ty3 Gag3 and Gag3 processing products in WCEs were quantified using a LAS-4000 Fuji imaging system and Multigauge V3.0 analysis software. This level was normalized to the Pgk1 loading control, and these values were normalized to the wt Ty3 protein signal. Two transformants of each sample were quantified and averaged, and a representative blot is shown. α-CA, anti-capsid. α-Pgk1, anti-Pgk1. **B.** Ty3 RNA levels. Two independent transformants were analyzed by RT-qPCR using pairs of primers that annealed in *GAG3* and in *ACT1* RNA. RT-qPCR was performed in the linear concentration range for two independent transformants, each in triplicate. Triplicates were

averaged and Ty3 *GAG3* signals were normalized to those of *ACT1*. The average normalized *GAG3* signal of the two transformants is presented. Ctl, uninduced control. **C.** RNAs containing Ty3 UTRs have lower Ty3 protein/RNA ratios. The ratio of Ty3 protein/RNA (determined as in A and B) was determined for cells expressing Ty3 RNA variants. UTRs, grey shaded boxes; hUTRs, white boxes.

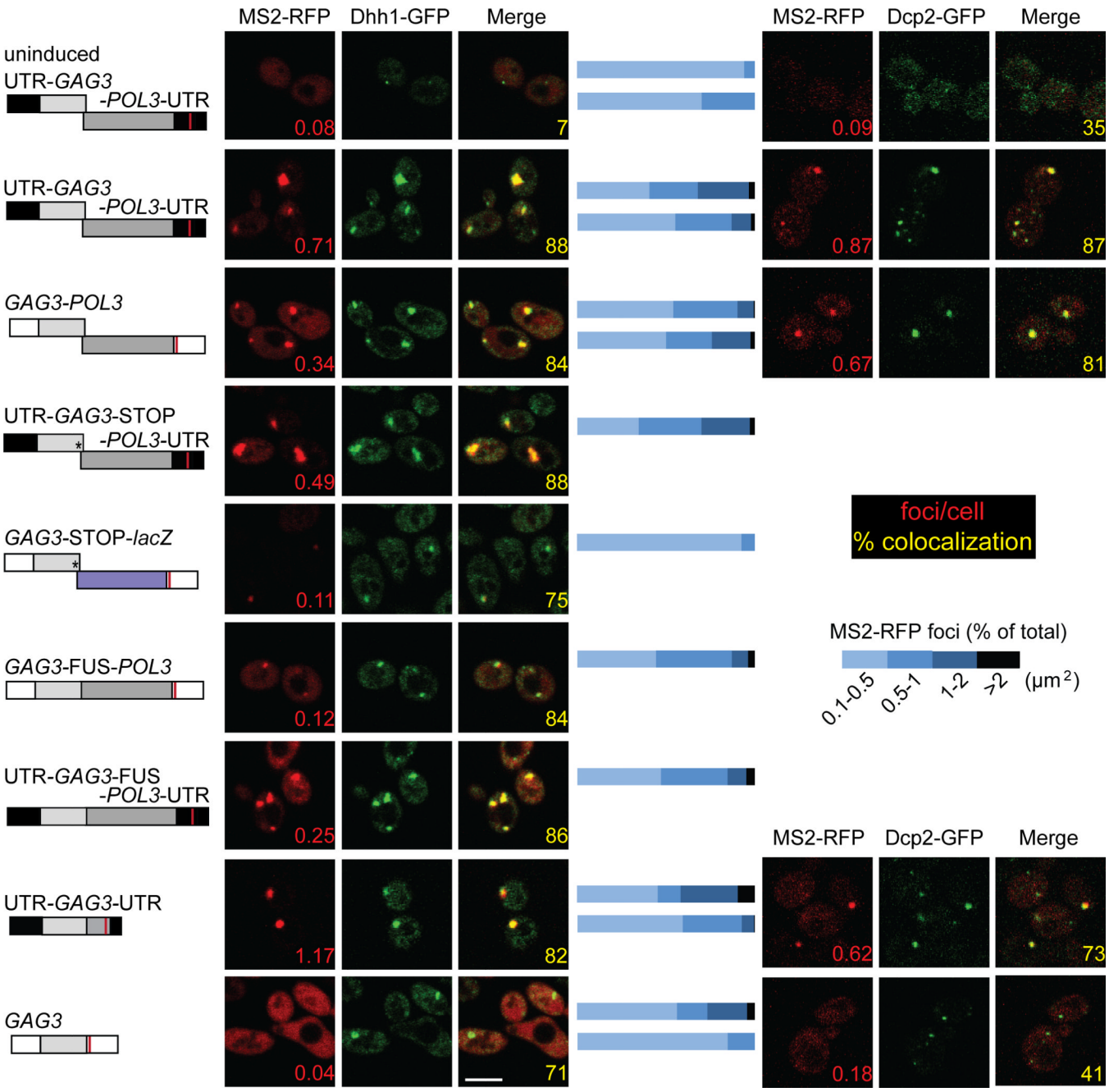

Figure 3. Ty3 sequences in UTRs and *POL3* independently mediate localization to foci with PB reporters

Confocal fluorescence microscopy localization of Ty3-related RNAs. BY4741 expressing the reporter protein Dhh1-GFP or Dcp2-GFP was transformed with Ty3-MS2 and MS2-RFP expression plasmids and induced as described in Methods. Images are representative of two independent transformants of each type. A minimum of 150 MS2-RFP-expressing cells were analyzed for each variant. The galactose-induced Ty3-MS2 RNA is indicated schematically to the left of the fluorescence image (for details see Fig. 1 legend). The top row of panels depict cells that were uninduced, while in the rest of the panels show induced cells. Inset numbers in red indicate number of RFP foci (greater than 0.1μm^2)/MS2-RFP-expressing cells. Inset numbers in yellow indicate the percentage of MS2-RFP foci overlapping Dhh1-

GFP or Dcp2-GFP reporter foci. The scale bar is 5 μm. Horizontal bars between images: relative size distribution of MS2-RFP foci measured as area of foci (in μm^2). Upper bar of pair: Dhh1-GFP; lower bar: Dcp2-GFP. Scale (white bar) is 5 μm. Data processing was as described in Methods.

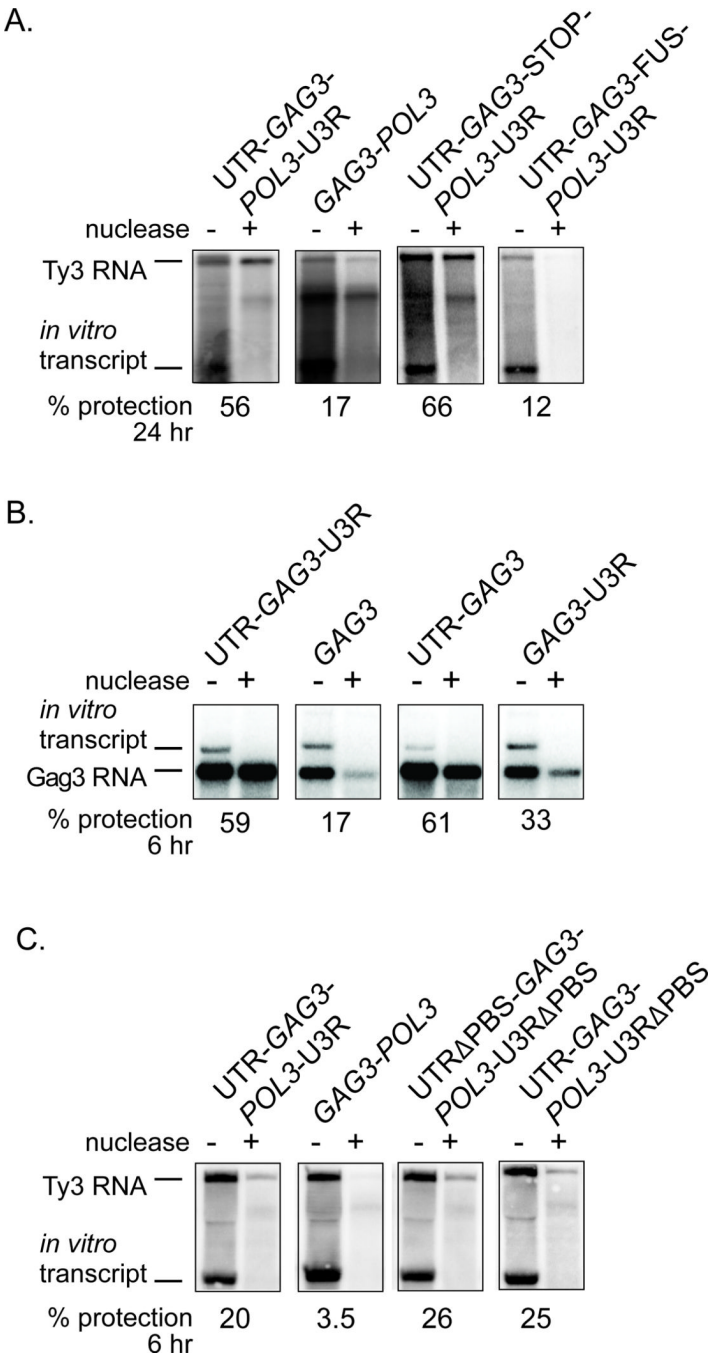

Figure 4. Packaging of variant Ty3 RNAs into VLPs

Extracts of cells expressing Ty3 RNAs were prepared under non-denaturing conditions after 24 h (A) or 6 h (B and C) of galactose induction. An equal amount of *in vitro* transcribed, truncated Ty3 RNA (Ty3 runoff RNA) was added to each WCE to monitor nuclease digestion. Nuclease treatment was performed as described in Methods. Northern Blots were probed with a ^{32}P-labeled DNA fragment specific to Ty3 RNA and quantified with Quantity One Software (Bio-Rad, Hercules, CA). Two or more independent experiments were performed for each Ty3 RNA variant, and a representative example is shown. % protection represents the amount of nuclease-treated RNA divided by the amount of untreated RNA. **A.**

Localization of Ty3 RNA with PB proteins in foci does not insure packaging. **B.** The 5' UTR of Ty3 is sufficient to direct packaging of short *GAG3*-containing RNAs. **C.** Deletion of the bipartite PBS sequences does not disrupt full-length Ty3 RNA packaging.

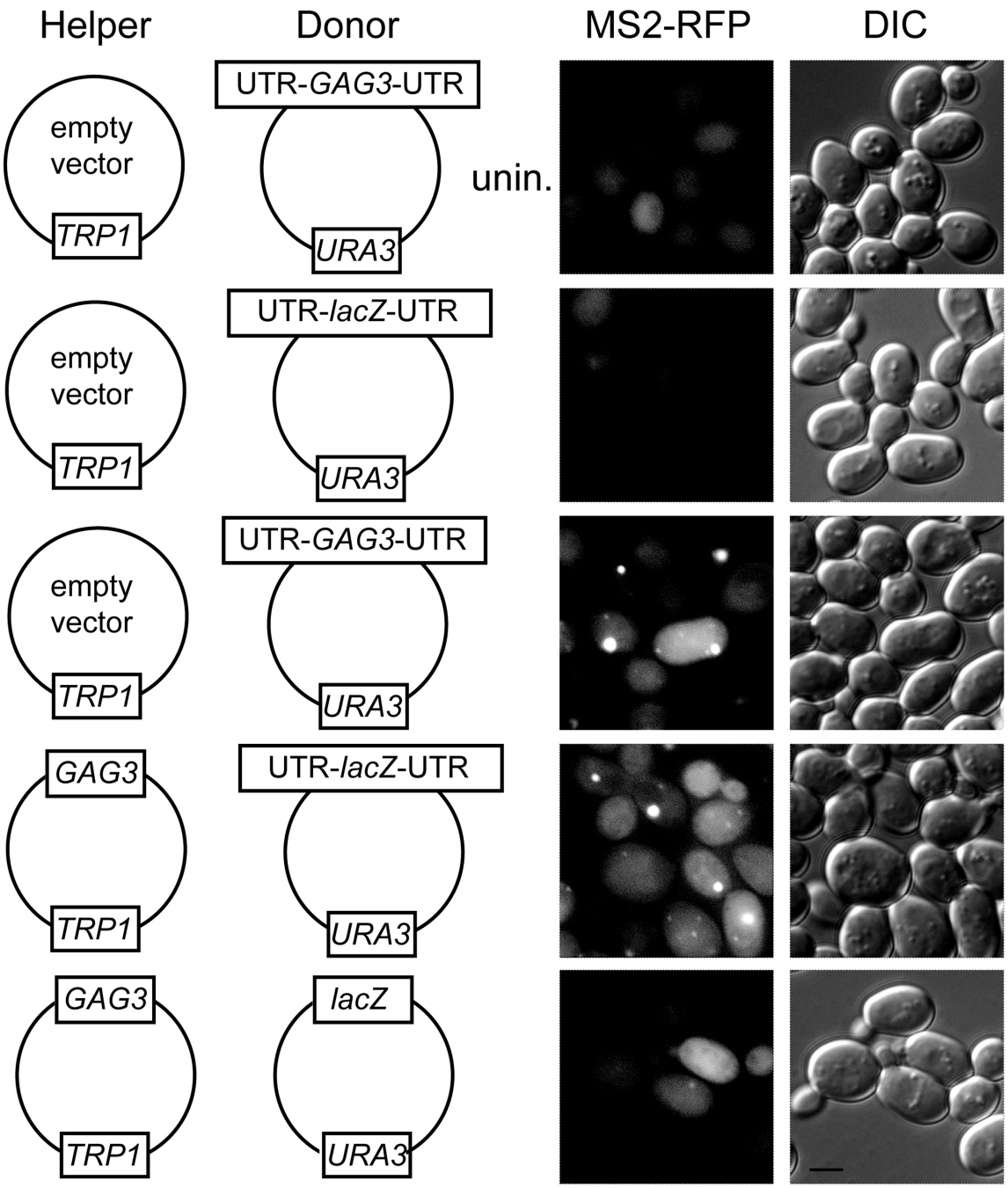

Figure 5. Gag3 packages Ty3 UTR-containing RNA *in trans*

Yeast strain BY4741 containing *trp1Δ* was transformed with a low-copy vector plasmid carrying *lacZ* with Ty3-specific UTRs or hUTR; these were co-transformed with low-copy helper plasmid carrying *GAG3* with hUTRs and low-copy plasmid carrying MS2-RFP reporter (see Table 1 and Methods). Samples were induced by addition of galactose and allowed to grow for 6 h (see Methods). The scale bar is 5 μm.

Table 1

Plasmids

Plasmid	Plasmid Contents	Genetic Markers (Base Plasmid)	Ty3 Sequences (in Ty3 DNA, nt#)	Reference
pYES2.0	empty vector	*URA3*, 2μ	None	Invitrogen
pRS315	empty vector	*LEU2, ARS4/CEN6*	"	(Sikorski & Hieter, 1989)
pRS314	empty vector	*TRP1, ARS4/CEN6*	''	(Sikorski & Hieter, 1989)
pDLC201 *	Full-length, gal-inducible Ty3 element; no MS2 binding sequence	*URA3*, 2μ	123–5351	(Hansen et al., 1988)
pSBS2183	Ty3-MS2 (UTR-*GAG3-POL3*-UTR-MS2)	*URA3*, 2μ (pDLC201)	"	(Beliakova-Bethell et al., 2006)
pNB2241	*GAG3-POL3*-MS2	*URA3*, 2μ (pYES2)	406–5057	This work
pNB2355	UTR-*GAG3*-STOP-*POL3*-UTR-MS2	*URA3*, 2μ (pDLC201)	123–5351	"
pNB2996	*GAG3*-STOP-*lacZ*-MS2	*URA3*, 2μ (pYES2)	406–1288	"
pNB2995	*GAG3*-FUS-*POL3*-MS2	"	406–5057	"
pNB2354	UTR-*GAG3*-FUS-*POL3*-UTR-MS2	*URA3*, 2μ (pDLC201)	123–5351	"
pNB3027	UTR-*GAG3*-UTR-MS2	*URA3*, 2μ (pYES2)	223–1288, 5012–5351	"
pNB2242	*GAG3*-MS2	"	406-1288	"
pNB3026	UTR-*GAG3*-MS2	"	223–1288	"
pNB3007	*GAG3*-UTR-MS2	"	406–1288, 5012–5351	"
pNB2629	$MET25_p$ -MS2-RFP-*CYC1* TT	*LEU2, ARS4/CEN6* (pRS315)	None	"
pNB2259	*LacZ* MS2	*URA3* 2μ, (pYES2)	None	''
pNB2247	5'UTR-*LacZ*-UTR	*URA3*, 2μ (pDLC201)	123–415, 5012–5351	
pNB2788	GAL-*GAG3*-CYC TT	*TRP1, ARS4/CEN6* (pRS314)	406–1288	''
pKC3700	Ty3-MS2 (UTR-*GAG3-POL3*-UTR-MS2)	*URA3*, 2μ (pYES2)	223–5351	"
pKC3702	UTRΔPBS-*GAG3-POL3*-UTRΔPBS-MS2)	*URA3*, 2μ (pYES2)	223–5351, Δ343–350 Δ5169–5196	"
pKC3703	UTR-*GAG3-POL3*-ΔPBSUTR-MS2)	*URA3*, 2μ (pYES2)	223–5351, Δ5169–5196	"

* Sequences of plasmids pDLC201 and below are available in Supplemental Materials